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**Scent of danger: floc formation of a freshwater bacterium induced  
by supernatants from a predator-prey coculture**

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Running Title: Bacterial floc formation in response to protistan predation

## ABSTRACT

We investigated predator-prey interactions in a model system consisting of the bacterivorous flagellate *Poterioochromonas* sp. strain DS and the freshwater bacterium *Sphingobium* sp. strain Z007. This bacterial strain tends to form a subpopulation of grazing-resistant microscopic flocs, presumably by aggregation. Enhanced formation of such flocs could be demonstrated in static batch culture experiments in the presence of the predator. The ratio of aggregates to single cells reached  $>0.1$  after 120 h of incubation in an oligotrophic growth medium. The inoculation of bacteria into supernatants from cocultures of bacteria and flagellates (grown in oligotrophic or in rich media) also resulted in a substantially higher floc formation than in supernatants from bacterial monocultures only. After separation of supernatants on a C18 cartridge the aggregate-inducing activity could be assigned to the 50% aqueous methanolic fraction, and further separation of this bioactive fraction could be achieved by HPLC. These results strongly suggest the involvement of one or several chemical factors in the induction of floc formation in *Sphingobium* sp. strain Z007, possibly released into the surrounding medium by flagellate grazing.

**Key words:** *Sphingobium*, *Poterioochromonas*, induced floc formation, grazing, predator-prey system

## INTRODUCTION

Interactions between free-living aquatic bacteria and predatory flagellates are determined by the balance between bacterial cell growth and mortality rates (1, 8, 23). High grazing mortality has favoured the evolution of various bacterial counter-strategies, such as small cell sizes, high-speed motility, or the production of toxins and other growth inhibitors (for review see, e.g., (25)). The particle uptake abilities of predators set tight constraints to the prey size that is preferentially ingested. As a consequence, protist-inedible filamentous cells may accumulate in heavily grazed freshwater bacterial communities, as well as other complex morphologies such as aggregates and microcolonies that are beyond the prey size spectrum of the predators (14, 18, 27). Such formation of grazing-resistant flocs at high protistan foraging is known both from static and continuous culture (13, 26) and from enrichments of natural waters (17).

A shift towards cell aggregates or microcolonies might simply be a result of the elimination of single celled morphotypes (13) but could also reflect an active response of bacteria to the presence of predators. Two non-exclusive causes can be envisaged for the enhanced growth of cells in aggregated form at such conditions. For one, higher floc formation might be a consequence of higher bacterial growth rates due to the release of additional substrates and nutrients by the predator (5, 8, 14, 36). Secondly, such growth behaviour might be induced by a chemical factor. Inducible morphological defence due to compounds released by the predators is common in other planktonic organisms, e.g. the spine induction in rotifers (12) and daphnids (20, 34), or the formation of grazing-resistant colonies by *Scenedesmus* (16). The exact nature and molecular action of most of these substances remains unknown, also because of the difficulty of establishing an appropriate bioassay to rapidly detect the effective fraction and to further characterize such compounds (29).

Recently, the formation of grazing-resistant filaments in the presence of a grazer was demonstrated for a *Flectobacillus* sp. strain in chemostat experiments (7). Since the predators were kept spatially separated from filament-forming cells (inside dialysis bags) this morphological shift was interpreted as indication for the action of kairomones. Such a continuous culture approach is complex and rather inconvenient for a subsequent chemical identification of the inducing factor by bioassay guided fractionation: Depending on flow rate and vessel size, continuous cultivation will yield relatively large volumes to be processed, and a single experiment in the above described study lasted for 10-20 days (7). By contrast, a bioassay based on static batch cultures would be experimentally simple, rapid, and it could be set up in a highly parallel manner in small volumes. However, neither the appropriate organisms nor conditions for a batch culture model of chemically induced morphological grazing resistance are as yet available. So far, the formation of aggregates/microcolonies (15), but not of filaments (7, 14) in the presence of a predator was demonstrated in batch culture. Batch culture experiments with a bacterial strain spatially separated from its predator showed that such cell aggregation may be induced both by growth state and by conspecific chemical cues (2). Thus, aggregate forming bacteria seem to be the more promising target in search of a grazing-related morphogenetic factor in static batch culture.

We sought to establish a batch culture bioassay for the detection and first tentative characterization of (one or several) chemical factors that would affect bacterial floc formation in a model predator-prey system. For this purpose, the growth behaviour of a freshwater bacterial isolate was investigated in direct contact experiments with the flagellate *Poterioochromonas* sp. strain DS and in supernatants derived from these cocultures.

## MATERIALS AND METHODS

**Bacterial and flagellate strains.** *Sphingobium* sp. strain Z007 was originally isolated from the surface water of Lake Zürich (Switzerland) (3). It is a rod-shaped moderately fast growing bacterium with the ability to form flocs (subsequently referred to as aggregates, but potentially also microcolonies, see discussion). The partial sequence of the 16S rRNA gene of strain Z007 was deposited in the EMBL Nucleotide Sequence Database (accession number FN293045). *Sphingobium* sp. Z007 was grown in DSMZ 7 medium (yeast extract: 1 g/L; glucose: 1 g/L; peptone: 1 g/L; DSMZ: German Collection of Microorganisms and Cell Cultures; Braunschweig, Germany) in 300 ml Erlenmeyer flasks at 18°C in the dark.

Axenic cultures of the bacterivorous flagellate *Poterioochromonas* sp. strain DS (3) were grown in ‘undefined *Ochromonas*-medium” (yeast extract: 1 g/L; meat extract: 1 g/L; glucose: 1 g/L; peptone: 1 g/L; Culture Collection of Algae (SAG) at the University of Göttingen, Germany). The flagellate cultures were kept in the dark at 18°C. They were additionally fed once per week with heat-killed cells ( $1 \times 10^7$  cells/ml; pre-incubated at 70°C for 2 h) of *Flectobacillus major* DSMZ 103.

All microbial strains were transferred into fresh medium 48 h before starting the experiments. During that period *Poterioochromonas* sp. strain DS cultures were no longer fed with heat-killed bacteria. After this period, no cells of *F. major* DSMZ 103 were detectable by epifluorescence microscopy. When *Sphingobium* sp. strain Z007 was used a second time during experiments with supernatants the culture was again freshly inoculated 48 h prior to use.

Two different media were used for the experiments. Initial co-cultivation experiments were carried out in a nutrient poor oligotrophic medium (artificial lake water, ALW). The concentrations of macroelements were chosen according to Zotina

et al. (39). Additionally, the following ingredients were added:  $\text{NH}_4\text{Cl}$  (30  $\mu\text{M}$ ),  $\text{K}_2\text{HPO}_4$  (322 nM),  $\text{NH}_4\text{Fe-Citrate}$  (110 nM Fe), L-Arg  $\times$  HCl (5 nM), Glucose (50 nM), L-Glu (15 nM), L-His  $\times$  HCl (7.5 nM), L-Met (7.5 nM), Thiaminiumdichloride (7.5 nM), Biotin (7.5 nM), and Vitamin  $\text{B}_{12}$  (7.5 nM). Later experiments with supernatants were carried out both in ALW and in DSMZ 7 medium (described above). Based on the outcome of the assays with whole supernatants only DSMZ 7 medium was used for the subsequent supernatant fractionation assays.

**Direct contact experiments.** Direct contact experiments were run in 300 ml Erlenmeyer flasks in a final volume of 50 ml medium at 18°C in the dark. Three batch cultures were inoculated with *Sphingobium* sp. strain Z007 ( $1.5 \times 10^6 \text{ ml}^{-1}$ ) and *Poterioochromonas* sp. strain DS ( $1.5 \times 10^3 \text{ ml}^{-1}$ ). As controls, triplicate batch cultures were only inoculated with *Sphingobium* sp. strain Z007 ( $1.5 \times 10^6 \text{ ml}^{-1}$ ) (Fig. 1, first panel). Subsamples of 1 ml were taken at six different time points (0, 24, 48, 72, 96, and 120 h) and fixed with glutaraldehyde (final concentration, 3%). Samples were stored at 1 °C until analyzed by flow cytometry (within 24 - 48 h).

**Supernatant experiments.** Additional direct contact experiments and pure bacterial batch cultures were prepared in ALW and DSMZ 7 media as described above in order to produce sufficient volumes of supernatants. All treatments were run in triplicates at 18 °C in the dark. Supernatants were produced after 72 h, 96 h, and 120 h of incubation time in ALW or after 120 h in DSMZ 7 medium. For this, the cultures were first centrifuged three times for 15 min at  $7400 \times g$  under sterile conditions. The pellet was subsequently discarded and the supernatants were frozen at -20 °C for one hour (Fig. 1, second panel) in order to destroy any potentially remaining flagellate cells. Immediately after thawing, the supernatants were filled in 24-well plates and inoculated with  $1.5 \times 10^6 \text{ ml}^{-1}$  *Sphingobium* sp. strain Z007 (the bacterial culture at this time had an age of 48 h, as described above). The final

volume in each treatment was 1.5 ml. The plates were then incubated in the dark for 24 h. Subsamples (500 µl) were taken after 24 h, fixed with glutaraldehyde (final concentration 3%), and immediately analysed by flow cytometry. The pH and dissolved organic carbon (DOC) concentrations of supernatants from the experiments with DSMZ 7 medium were determined both in the control treatments of *Sphingobium* sp. strain Z007 only and in cocultures of bacteria with *Poterioochromonas* sp. strain DS (initial abundances,  $1.5 \times 10^3 \text{ ml}^{-1}$ ). For DOC analyses the samples were centrifuged, the supernatants were transferred into glass vials (prewashed with 2 M HCl and sterile deionized H<sub>2</sub>O) and analysed using a TOC-5000 Total Organic Carbon Analyzer (Shimadzu, Japan).

**Fractionation experiments – C18 cartridge.** Supernatants were produced in triplicates as described above from controls (bacteria only) and from cocultures with initial concentrations of  $1.5 \times 10^3 \text{ ml}^{-1}$  cells of *Poterioochromonas* sp. strain DS (DSMZ 7 medium, 120 h of incubation in the dark). After centrifugation and ‘freeze-thawing’ the supernatants (20 ml) were loaded onto a pre-equilibrated C18 cartridge (Marchery-Nagel, Düren, Germany). The direct eluate was collected and is subsequently referred to as ‘hydrophilic fraction’. Substances bound to the cartridge were first washed out with 10% aqueous (aq.) methanol (MeOH), followed by 50% aq. MeOH, and finally by 100% MeOH. After evaporation of the solvents in a rotavapor (35 mbar; 30°C) the residues were re-dissolved in fresh DSMZ 7 medium, and frozen at -20 °C for one hour (Fig. 1, third panel). Next, all fractions were filled into 24-well plates and inoculated with  $1.5 \times 10^6 \text{ ml}^{-1}$  *Sphingobium* sp. strain Z007 (final volume 1.5 ml). The 24-well plates were then incubated in the dark at 18 °C. Samples (500 µl) were taken after 48 h, fixed with glutaraldehyde (final concentration 3%), and immediately analysed by flow cytometry.



In one experiment fresh DSMZ 7 medium was loaded onto the C18 cartridge as an additional control. Bacterial aggregate formation did not differ between treatments amended with such fractionated fresh medium or with fractionated supernatants of the bacterial cultures (data not shown).

**Fractionation experiments – HPLC.** Supernatants were produced in triplicates as described above from controls (bacteria only) and from cocultures with initial concentrations of  $1.5 \times 10^3 \text{ ml}^{-1}$  cells of *Poterioochromonas* sp. strain DS (120 h of incubation in the dark). The supernatants were separated by C18 cartridge and again tested for their ability to enhance aggregate formation in *Sphingobium* sp. strain Z007 in order to verify the results of the above described experiments. The aggregate inducing fractions derived from the supernatants of triplicate cocultures (50% aq. MeOH) were then further separated (Fig. 2, fourth panel). HPLC fractionation was performed on a Shimadzu 10AVP system with a photodiode array detector and a C18 ODS-A reversed phase column (250 × 4.6 mm, 5 µm particle size; YMC Europe GmbH, Dinslaken, Germany). The flow rate was 1 ml/min. Solvent A was composed of UV-treated deionized H<sub>2</sub>O and 0.05% trifluoroacetic acid (TFA; Fluka, Switzerland) and solvent B was acetonitrile and 0.05% TFA. A linear increase was applied (solvent B from 20% to 100% in 41 min). The injection volume was 500 µl. Four subfractions were collected according to retention time (1 – 11 min, 11 – 21 min, 21 - 31 min, 31 - 41 min). The subfractions of five runs were combined for further analyses. The solvents were evaporated using a vacuum evaporator at 30°C, the residues were re-dissolved in 2.5 ml of fresh DSMZ 7 medium, and the pH was adjusted to 7.2. Subsequently, the subfractions were frozen at -20°C for one hour. After thawing the subfractions were filled into 24-well plates and inoculated with  $1.5 \times 10^6 \text{ ml}^{-1}$  *Sphingobium* sp. strain Z007 (final volume 1.5 ml). The 24-well plates were then incubated in the dark at 18 °C. Samples (500 µl) were taken after 48 h, fixed

with glutaraldehyde (final concentration 3%), and immediately analysed by flow cytometry.

**Flow Cytometric Enumeration.** Samples for flow cytometry were stained with 4',6-diamidino-2-phenylindole (DAPI) (final concentration  $1 \mu\text{g ml}^{-1}$ ) for 10 min in the dark, and analyzed using the Influx V-GS cell sorter (Becton Dickinson Inc., San Jose, Ca.). A blue (Coherent, Sapphire, 200 mW, 488 nm) laser was used for detection of light scatter and the autofluorescence of flagellates, and a UV-laser (Lightwave Electronics, CY-PS, 60 mW, 355 nm) for DAPI fluorescence. If required, samples were diluted with  $0.2 \mu\text{m}$  pre-filtered deionised water to avoid particle coincidence. To convert the flow cytometry counts to cell numbers a defined amount of microspheres (latex beads, Polyscience, FlowCheck<sup>®</sup> High Intensity Green Alignment, diameter  $1.0 \mu\text{m}$ ) were added to the samples. Bead working solutions were freshly prepared daily. *Poterioochromonas* sp. strain DS was identified using  $90^\circ$  light scatter vs. green fluorescence (531 nm). Single cells of *Sphingobium* sp. strain Z007 were identified using  $90^\circ$  light scatter vs. DAPI fluorescence (Fig. 2, SC). Due to the markedly larger size and up to three orders of magnitude lower abundances the aggregates were counted separately applying different cytometric settings. Large cell aggregates (Fig. 2, Agg) were operationally defined by their fluorescence and scatter properties, i.e., signals with equal or higher scatter / fluorescence than that of *Poterioochromonas* sp. strain DS (Fig. 2, P) at comparable instrument settings (as determined by fluorescent microspheres). This approach allowed for the reproducible quantification of aggregates above a fixed size threshold and the reliable distinction of aggregates from single cells, which was also verified by cell sorting and microscopic inspection.

**Statistical Analyses.** Statistical analyses were performed using the software SPSS 16 (SPSS Inc., Chicago, Ill.). The following hypotheses were tested: (i) the fractions of bacterial aggregates in direct contact experiments after 72 h of incubation were higher in cocultures of bacteria and flagellates than in bacterial cultures (one-sided paired t-test on arcsine transformed pooled data from timepoints 72, 96 h, and 120 h); (ii) the numbers of bacterial aggregates after 120 h of incubation in supernatants of coculture were higher than in supernatants of pure bacterial cultures (one-sided Mann-Whitney U-Test, performed separately for supernatants from DSMZ and ALW media); (iii) the fractions separated by the C18 cartridge and the subfractions separated by HPLC differed in their potential to induce aggregate formations (two-sided Kruskal-Wallis ANOVAs, followed by Scheffé post-hoc tests on arcsine transformed data).

## RESULTS

**Direct contact experiments.** The number of single cells of *Sphingobium* sp. strain Z007 in the controls increased over time from  $0.8 \times 10^6 \text{ ml}^{-1}$  to  $6.0 \times 10^6 \text{ ml}^{-1}$  after 48 h of incubation. The increase in bacterial single cell abundances in the cocultures with *Poteroochromonas* sp. strain DS diminished in parallel with the rise of the flagellate population ( $0.14 \times 10^6 \text{ ml}^{-1}$  after 72 h) (Fig. 3A). At 48 h the abundance of aggregates was similar in both treatments. However, concomitant with the increasing concentrations of flagellates the number of aggregates almost doubled during the following 24 h and remained stable thereafter until the end of the experiment. Comparable ratios of aggregates to single cells were present in the controls and in the cocultures during the first 24 h of the experiments. Minor differences could already be observed after 48 h and became increasingly more

pronounced between 72 h until the end of the experiment (Fig. 3B). During this later period the ratio of aggregates to single cells in treatments with direct contact between bacteria and flagellates were significantly ( $P<0.01$ , up to 20 times) higher than in the controls. After 120 h of incubation, the ratio of aggregates to single cells in the coculture reached more than 0.1. It should be noted that this value only represents the fraction of aggregates with at least the same size (i.e., side scatter value) as the flagellates (Fig. 2). Taking into account all aggregates that were clearly distinguishable from single cells in the cytograms this ratio reached a value of over one third of single cells in the coculture treatment after 120 h, but less than 0.04 in the control treatment.

**Supernatant experiments.** The numbers of aggregates formed after 24 h of incubation in supernatants from cocultures of bacteria and flagellates in ALW were always slightly (but not significantly) higher than in the controls (supernatants from bacteria only in ALW) (Fig. 4). This difference was most pronounced between supernatants from cultures or cocultures that had been incubated for 120 h. At the same time the numbers of single cells in these supernatants did not increase or even slightly decreased within 24 h.

By contrast, a clear increase in single cell abundances was observed in supernatants that were derived from either bacteria or from cocultures of bacteria and flagellates grown in DSMZ 7 medium (Fig. 4): This rise in cell numbers was roughly 7 times higher in the supernatants from the control treatments (bacteria only) than in those from the cocultures. At the same time the numbers of aggregates in the supernatants from the cocultures was significantly ( $P<0.01$ , approximately 60 times) higher than in those from the pure bacterial cultures, resulting in a >300 times higher ratio of aggregates to single cells (Fig. 4). The pH values were 7.5 in the supernatants from the control treatments (bacteria only, grown in DSMZ 7 medium),

and 7.1 in supernatants from the coculture with *Poterioochromonas* sp. strain DS. The DOC concentrations were 703 mg l<sup>-1</sup> in the supernatants of the control treatments and only slightly higher in those from the cocultures (841 mg l<sup>-1</sup>).

**Fractionation experiments.** In a next step several fractions derived from the separation of supernatants on a C18 cartridge were tested for their potential to induce aggregate formation in *Sphingobium* sp. strain Z007 (Fig. 5). Generally, the abundances of single cells were slightly but not significantly higher in treatments supplemented with fractions from the control supernatants than in those with added fractions from the coculture supernatants ( $4.3 \times 10^8 \pm 0.43 \times 10^8$  ml<sup>-1</sup> compared to  $3.7 \times 10^8 \pm 0.28 \times 10^8$  ml<sup>-1</sup>). At the same time the total abundances of aggregates were typically higher in the treatments supplemented with supernatant fractions from the cocultures (data not shown). However, only the 50% aq. MeOH fraction from the coculture supernatant induced a significant increase in aggregates, resulting in a significantly ( $P < 0.05$ ) higher ratio of aggregates to single cells compared to the controls.

In a final experiment this 50% aq. MeOH fraction derived from the supernatants of cocultures was further separated by HPLC (Fig. 6). All together four subfractions from this HPLC separation were tested in triplicate experiments for their potential to trigger aggregate formation in *Sphingobium* sp. strain Z007. Only one subfraction (eluted between 11 and 21 min under the conditions applied) induced a significant ( $P < 0.05$ ) increase in the proportions of formed aggregates in comparison with the other subfractions. This subfraction showed no absorption in the visible light spectrum (data not shown) and only low absorption in the UV spectrum at 210 nm (Fig. 6, lower panel).

## DISCUSSION

**Bacterial floc formation during flagellate grazing.** *Sphingomonadaceae* have been repeatedly isolated from freshwater habitats (11, 24) and could be enriched after addition of low concentrations of natural dissolved organic matter (9). These bacteria are widely distributed across freshwater habitats, where they may produce densities up to  $>10^6$  cells ml<sup>-1</sup> (28). *Sphingomonas* sp. and related bacteria have also been found in newly formed and aged freshwater organic aggregates (lake snow) (19, 31), and the planktonic *Sphingomonas natatoria* may even co-aggregate with other species in order to establish and expand its populations within freshwater biofilms (22). Such findings suggest that the *Sphingobium* sp. strain Z007 used in our study may represent an appropriate model organism to investigate the induction of aggregate/microcolony formation. It has the tendency to spontaneously form flocs even in pure cultures during particular phases of batch growth in rich media (2) and to a small extent also at the comparatively oligotrophic growth conditions in artificial lake water (Fig. 3).

The inoculation of flagellates into the batch cultures resulted in reduced abundances of single bacterial cells, and the rise in grazing pressure at higher flagellate densities was paralleled by a proportional increase of the aggregated bacterial subpopulation (Fig. 3). This clearly demonstrates a protection of this size class of aggregates against grazing by *Poteroiochromonas* sp. strain DS. Moreover, the high ratio of aggregates with a larger size than the flagellates to single cells in the oligotrophic artificial lake water medium (Fig. 3 B) points at the ecological relevance of aggregate formation in the presence of predators under close to natural growth conditions. Batch culture systems have been successfully used before in predator-prey interactions with freshwater bacterial isolates and axenic flagellates. Increased

formation of suspended microcolonies by *Pseudomonas* sp. MWH1 (15) and by two strains of beta-proteobacteria (13) were found in static batch cultures in the presence of *Ochromonas* sp. strain DS (now *Poterioochromonas* sp. strain DS (4)).

Various grazing-resistant bacterial morphologies have been observed in single strains and bacterial assemblages, such as microcolonies, aggregates, and filaments (13, 30, 32). While filamentous morphotypes are easily definable it is more difficult to distinguish between bacterial aggregates and microcolonies. Microcolonies originate from cells that do not separate after division whereas aggregates result from the encounter of freely dispersed single cells (15). Thus, both, aggregates and microcolonies might quickly increase in numbers during the early phase of batch growth. However, as soon as the abundances of single cells decline due to grazing, there should be little further increase in aggregate numbers (15), whereas no such limitation would apply for microcolonies. In our co-cultures the number of bacterial flocs doubled within 24 h after the flagellates had reached high densities (between 48 – 72 h) (Fig. 3), but remained stable thereafter when the numbers of single cell were low. This seems to suggest aggregation as major mechanism of floc formation. Moreover, the growth rate of single cells of *Sphingobium* sp. strain Z007 in the oligotrophic medium was low ( $0.04\text{ h}^{-1}$ ), which also speaks against the formation of bacterial flocs (typically consisting of 20 cells or more) by cell division only. Finally, microscopic inspections suggested that single cells of *Sphingobium* sp. strain Z007 were motile and would thus be able to actively assemble. Nevertheless, we cannot exclude that the bacterial flocs -tentatively classified as aggregates- were in parts also formed by cell division processes.

**Technical aspects of bioassay development.** An important step in the development of the bioassay was to produce supernatants that were entirely free of

flagellates. In the past, 0.2  $\mu\text{m}$  filtration has been applied for this purpose (6, 15). However, extractable substances from different membrane filters may by themselves induce morphological changes as was shown for the green alga *Scenedesmus obliquus* (21). Therefore, using filtration might lead to false positive or negative results. A combination of centrifugation and freezing of supernatant seems to be more appropriate to avoid any introduction of additional chemical components to the supernatants. A dilution of supernatants - as done in previous studies (6, 7, 15) - might moreover decrease the concentration of the trigger compounds. Since the treatment-specific response of bacteria were most clearly observed in supernatants from the rich medium than in those from the oligotrophic one (Fig. 4), the former were chosen for all subsequent fractionation experiments. However, it should be noted that the substantially higher numbers of single cells in these supernatants also led to generally lower relative proportions of aggregates than was observed when using artificial lake water.

In order to assess the relationship between aggregation and growth state it was, moreover, important to accurately determine the numbers of both single cells and aggregates over a large range of total cell concentrations (Fig. 2). Our flow cytometric assay provided a fast and precise means of simultaneously quantifying free single cells and an operationally well-defined population of aggregates in the same sample, e.g., those that exceed the approximate size of the predators, as estimated by their respective light scatter properties (Fig. 2). The sorting and microscopic inspection of different regions from cytograms moreover allows for a verification of the counting gates (and for additional analyses on subpopulations).

**Effects of supernatants on cell aggregation.** Chemical factors have been previously suggested as inducing agents of bacterial cell aggregation during



predation. For example, the feeding of *Poterioochromonas* sp. DS on *Sphingobium* sp. strain Z007 outside of dialysis bags lead to significantly higher proportions of bacterial aggregates inside the bags (2). In our experiments, the addition of supernatants from a 120 h old coculture of bacteria and flagellates incubated in oligotrophic artificial lake water medium to a pure culture of *Sphingobium* sp. strain Z007 resulted in the enhanced formation of aggregates compared to treatments with supernatants from bacterial monocultures (Fig. 4). The treatment specific enrichment of aggregates in supernatants from cocultures was particularly evident in the experiments using the rich DSMZ 7 growth medium (Fig. 4). In view of the comparable concentrations of DOC, the enhanced cell aggregation in the supernatants from the cocultures represent first evidence for the presence of one or several chemical factors that are involved in the stimulation of cell aggregation of *Sphingobium* sp. strain Z007, and that are possibly secreted by *Poterioochromonas* sp. strain DS as a consequence of bacterivory. Moreover, the considerably higher numbers of single cells formed in the supernatants from pure bacterial cultures (Fig. 4) suggest that this cell aggregation was not merely due to higher growth rates in supernatants from cocultures.

Several investigations have explored if morphological changes of bacteria can be induced by supernatants from batch cocultures of flagellates and bacteria (6, 7, 13, 15). So far no comparable observation of chemically induced bacterial floc formation has been reported. This may indicate that the findings from our model system cannot be generalized, and it is possible that aggregate or microcolony formation in other experimental predator prey systems might not have been triggered by chemical factors (7, 13, 15). Moreover, the visible development of a grazing resistant bacterial subpopulation might depend on the balance between bacterial

growth and grazing losses in the cocultures from which the supernatants were produced. The generation of active compounds is arguably linked to flagellate digestion. Thus, low initial concentrations of flagellates might only produce a relatively small amount of the aggregate-forming substance(s). At too high predator-prey ratios bacteria might be consumed too quickly and flagellates might be starved at the time point when the co-cultures are processed for supernatant production. We tested various initial predator-prey ratios prior to our study (data not shown). A ratio of 1:1000 cells resulted in the most significant increase of cell aggregation in the supernatant experiments and was, therefore, chosen for all successive experiments. Finally, chemical interactions between bacteria and flagellates might also be obscured by choosing different bacteria as food source for the predators (for the production of supernatants) and as test organisms (for subsequent examination of the effects). Various aquatic organisms exhibit morphological defence against predators that are only triggered by conspecific chemical cues (10, 33, 38). Enhanced aggregate formation in our tested strain was only induced by flagellate grazing on cells from the same species (2).

**Chemical nature of aggregate-inducing factor.** Supernatants of bacterial monocultures and of cocultures of bacteria and flagellates could be successfully fractionated using a C18 cartridge (Fig. 5). Since the fractionated supernatants were re-dissolved into fresh medium for these experiments, it is not possible to directly compare the level of aggregate formation in these assays with the level observed after addition of whole supernatants (that were likely depleted in substrates). Instead, we focused on the differences between the treatments amended with the individual fractions. Upon addition of the 50% aq. MeOH fraction from the coculture bacterial aggregate formation was significantly higher than in all other fractions from this

treatment type or from the supernatants of the pure bacterial cultures (Fig. 5). Subsequently, the 50% aq. MeOH fraction was further separated using common reversed phase HPLC methods, and one of these subfractions could again be shown to disproportionally induce cell aggregation (Fig. 6).

Under the conditions applied the aggregate inducing compound was of a moderate lipophilic nature. As corresponding absorption characteristics are missing, this compound most likely does not possess a chromophore or protein/peptide substructures. However, the absorption of very low concentrations of a highly potent active compound might be underestimated at our specific setup. Our findings are in line with what is known about the nature of comparable chemical factors from aquatic organisms. For example, the putative kairomones from fish and *Chaoborus* larvae are moderate lipophilic substances of low molecular weight and high thermal and pH-stability (35, 37), but their exact chemical nature remains unknown. While the results of our fractionation experiments are strong evidence for the involvement of a predation related chemical factor in induced floc formation of *Sphingobium* sp Z007, the elucidation of the chemical nature of this factor would go beyond the scope of this study. Since the here presented bioassay is relatively fast and can be performed in small volumes (1.5 ml) as compared to models that involve chemostats (7) or larger metazoan predators, it could provide a means for the future characterization of a factor that apparently modifies the growth behaviour of an aquatic bacterium.

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Legend to figures:

FIG. 1. Experimental Setup: *Sphingobium* sp. strain Z007 as control and *Sphingobium* sp. strain Z007 and *Poterioochromonas* sp. strain DS in direct contact experiments (first panel), after centrifugation and freezing in supernatant experiments (second panel), after first separation step on a C18 cartridge (third panel), and after further separation step on a HPLC C18 ODS-A reversed phase column (fourth panel).

FIG. 2. Cytogram (90° light scatter vs. UV fluorescence) of *Sphingobium* sp. strain 007 after staining with DAPI. The gate in the upper right corner of the graph comprises events that were counted as bacterial cell aggregates. Grey dots depict cells of *Poterioochromonas* sp. strain DS in the same sample. The inset shows the cytogram used for the counting of *Poterioochromonas* sp. strain DS (90° light scatter vs. green fluorescence). Agg: Aggregates; SC: single cells; P: *Poterioochromonas* cells.

FIG. 3. A: Time course of changes in abundances of *Poterioochromonas* sp. strain DS (grey triangles), and of single cells of *Sphingobium* sp. strain Z007 in controls (bacteria only, black circles) and in coculture with the flagellate (white circles) during batch culture incubation in artificial lake water medium.

B: Ratios of aggregates to single cells of *Sphingobium* sp. strain Z007 in control (black bars) and in coculture with the flagellate (white bars), and abundances of aggregates of *Sphingobium* sp. strain Z007 in controls (black squares) and in coculture with the flagellate (white squares). Error bars indicate the standard errors of three replicates. Asterisks: significantly higher than control treatments at  $P < 0.01$ .

FIG. 4. Abundances of aggregates at  $t_{24h}$  (upper panel) and differences in single cell numbers between  $t_{0h}$  and  $t_{24h}$  (lower panel) of *Sphingobium* sp. strain Z007 in the supernatants of pure bacterial cultures (black bars; SP) and in supernatants of cocultures with *Poteroochromonas* sp. strain DS (white bars; SP+P). Supernatants were derived from experiments that were performed using artificial lake water (ALW) and rich medium (DSMZ 7) and that were obtained from direct contact experiments at different time points (72 h, 96 h, 120 h). Error bars indicate the standard error of three replicates. Asterisk: significantly higher than other fractions at  $P < 0.01$ .

FIG. 5. Abundances of single cells, and ratios of aggregates to single cells of *Sphingobium* sp. strain Z007 after 48 h of incubation in DSMZ 7 medium amended with fractions obtained by C18 cartridge separation of supernatants from pure bacterial cultures (A1 and A2) and in supernatants from cocultures (B1 and B2). Error bars indicate the standard error of three replicates. Asterisk: significantly higher than other fractions at  $P < 0.05$ .

FIG. 6. Upper panel: Ratios of aggregates to single cells of *Sphingobium* sp. strain Z007 after 48 h of incubation in DSMZ 7 medium amended with four subfractions obtained by HPLC separation of the 50% aq. MeOH fraction of the supernatant of the bacteria and flagellate coculture. Error bars indicate the standard error of three replicates. Asterisk: significantly higher than other fractions at  $P < 0.05$ . Lower Panel: HPLC chromatograms (at 210 nm and 278 nm) of the 50% aq. MeOH fraction with marked collected fractions (Fr1-Fr4).

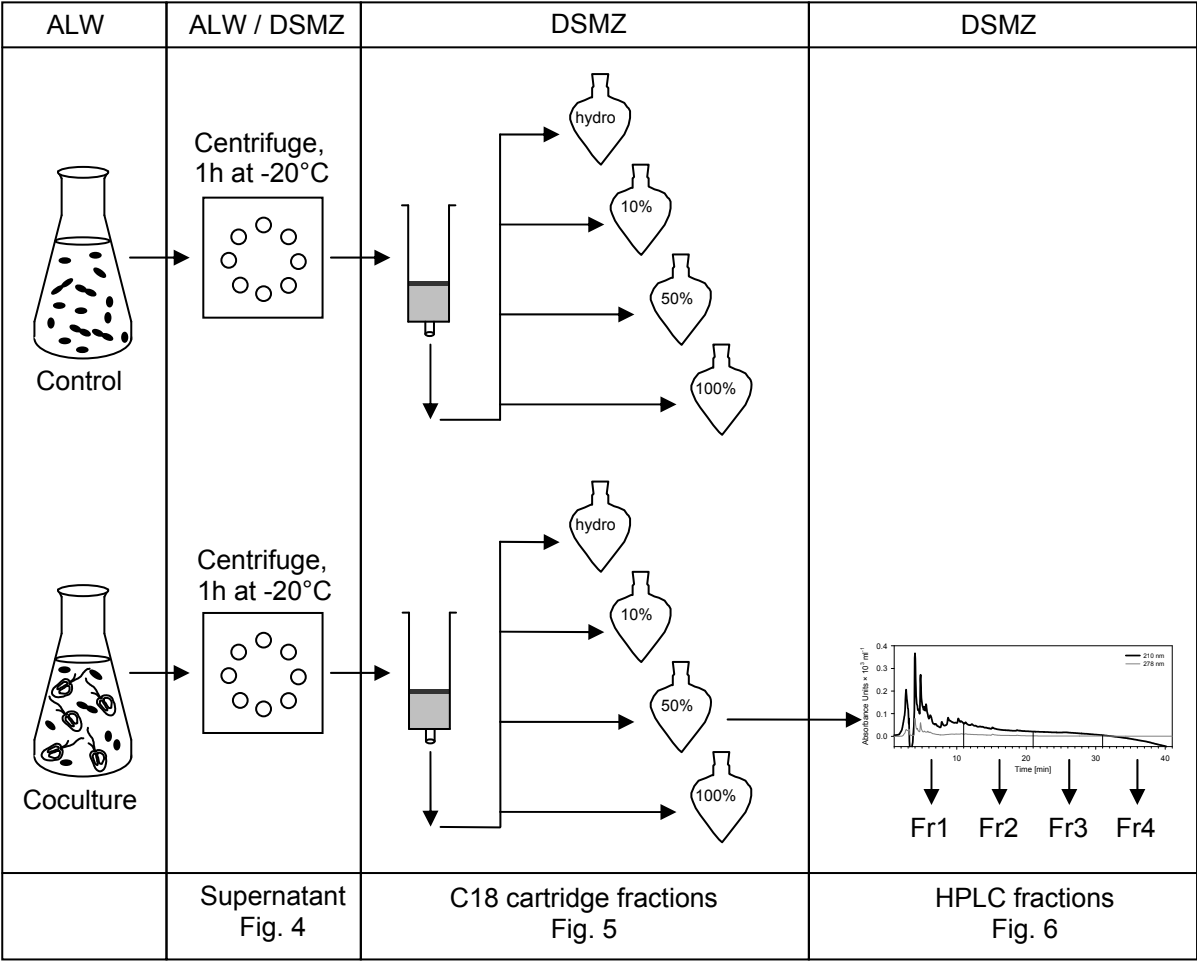


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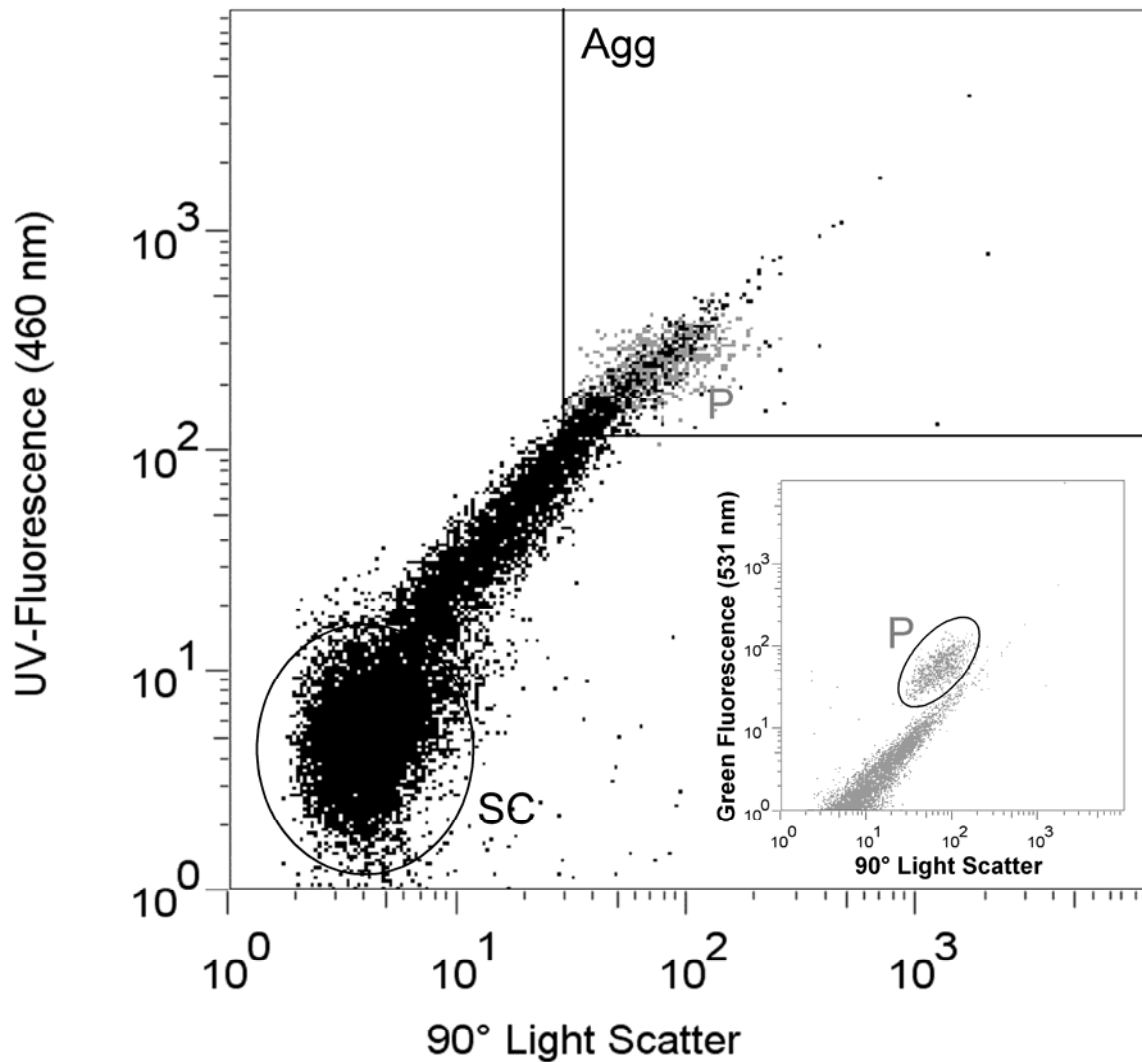


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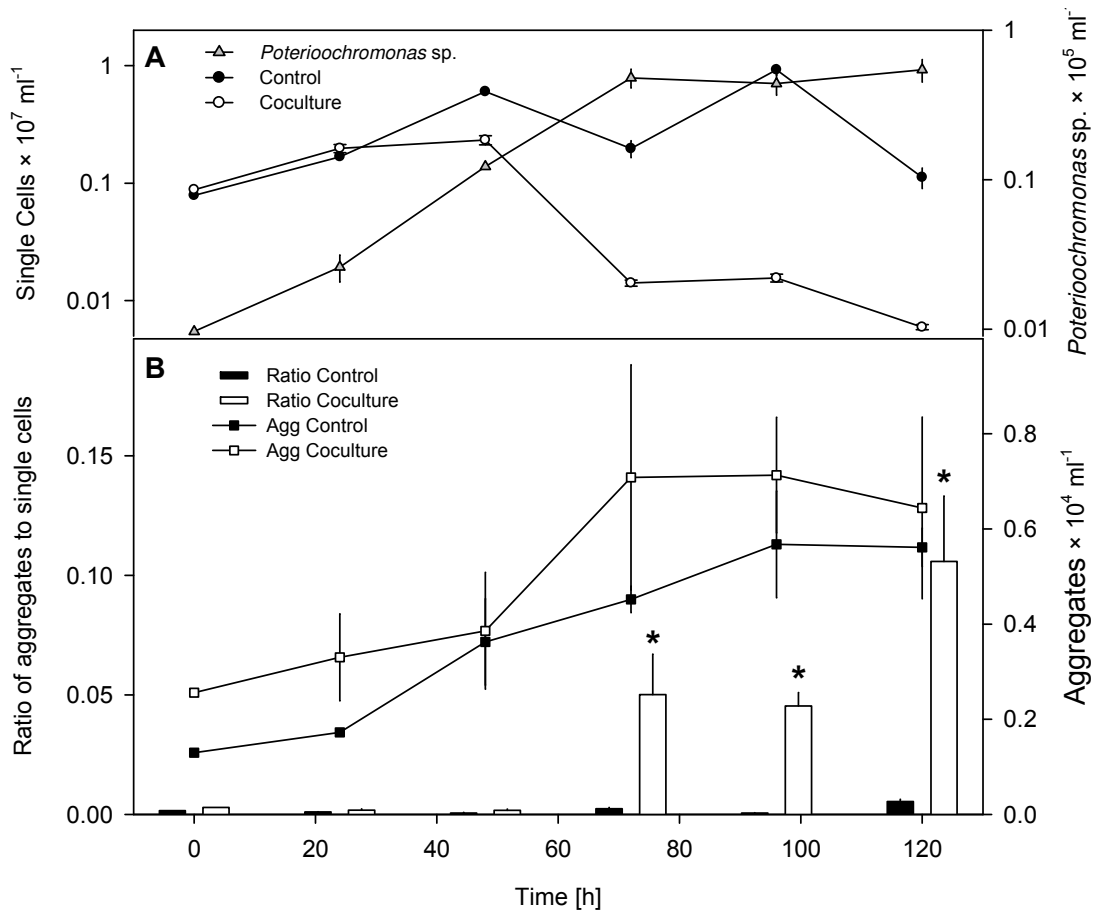


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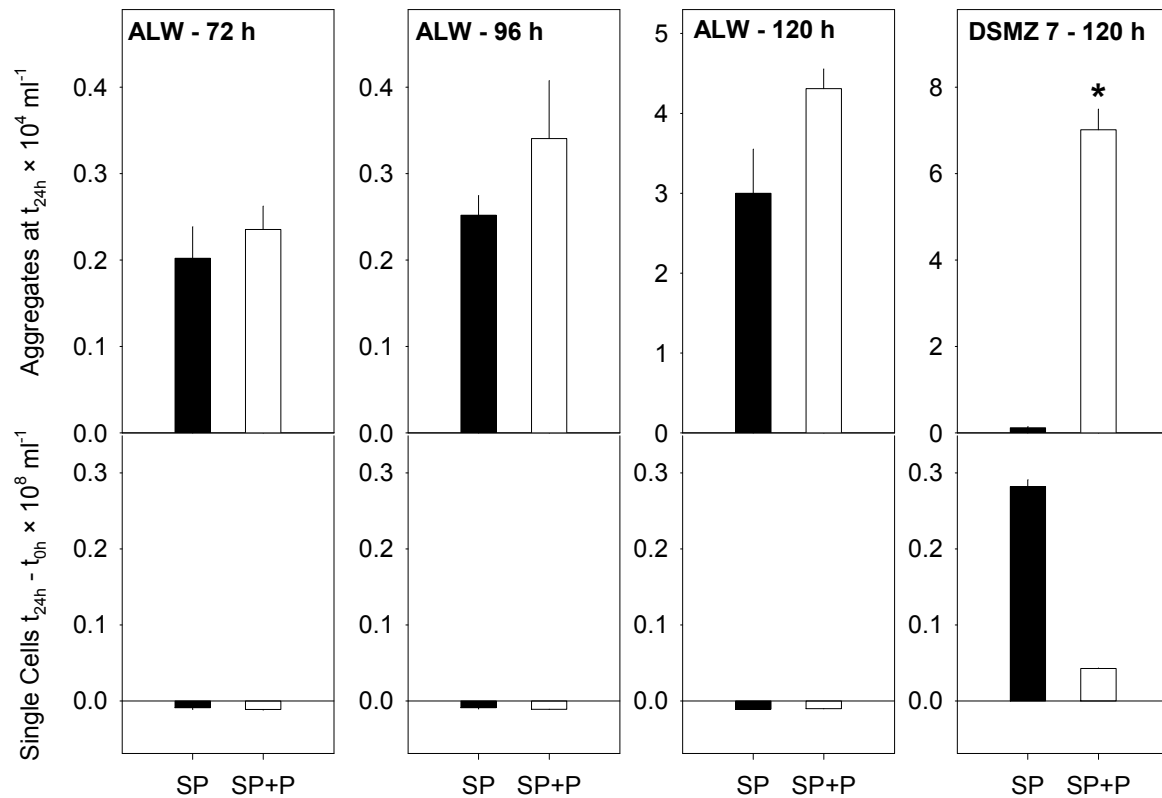


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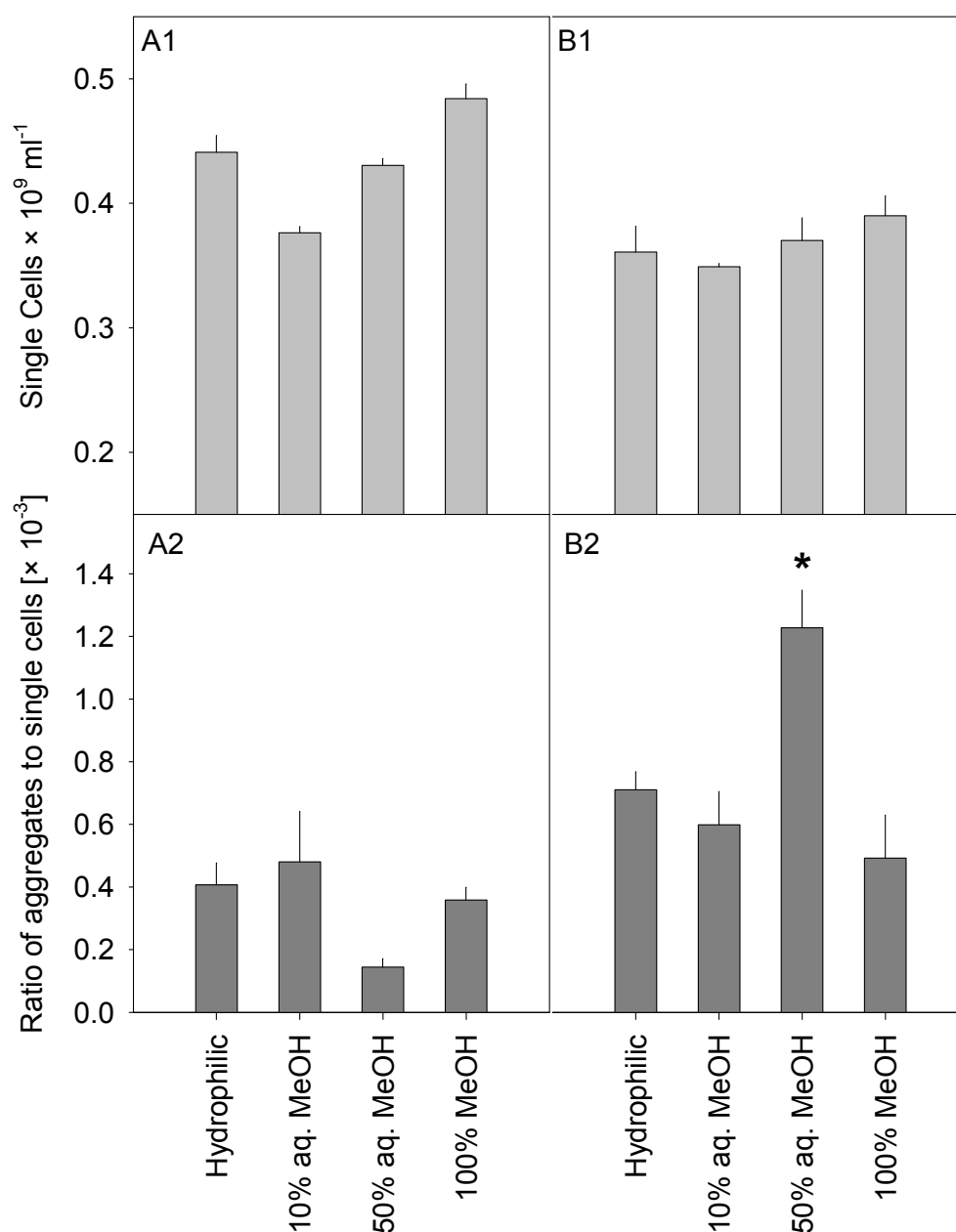


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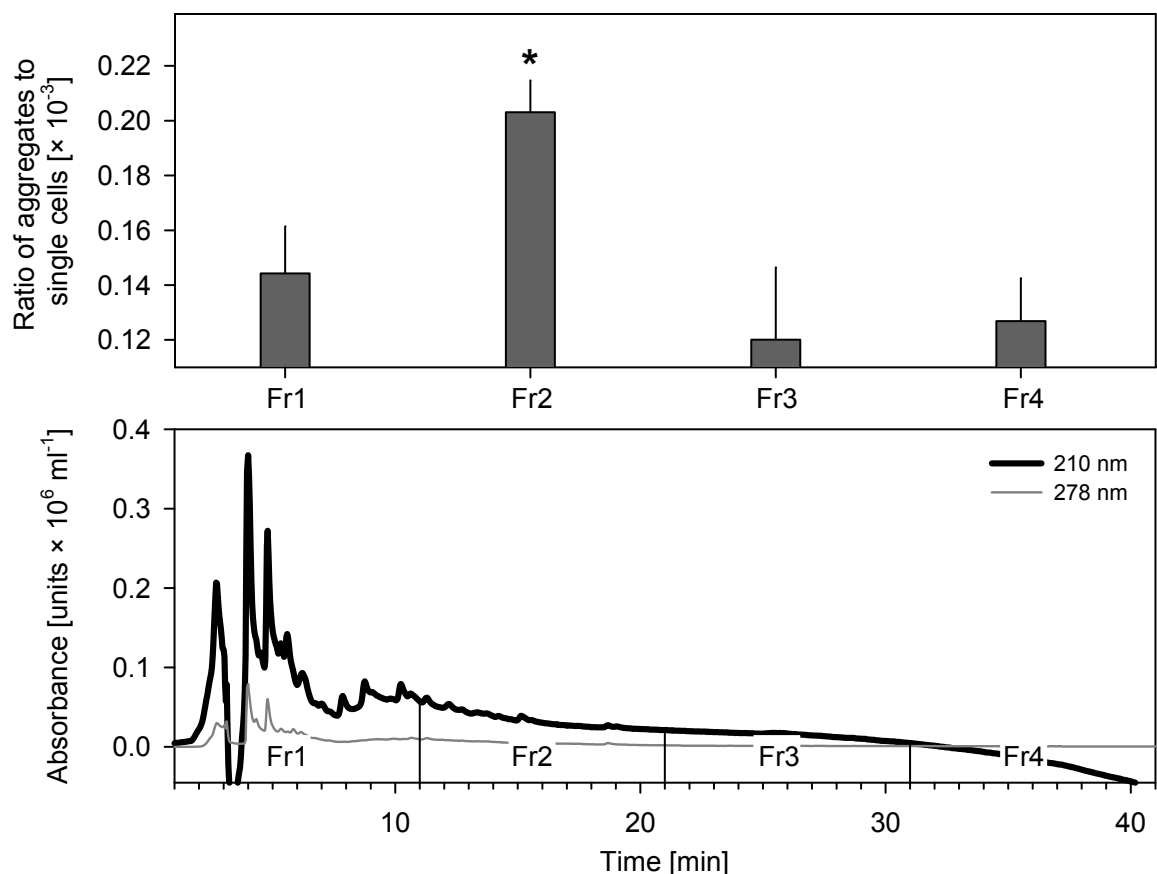


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